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Role in the selectivity of neonicotinoids of insect-specific basic residues in loop D of the nicotinic acetylcholine receptor agonist binding site

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Running title: Basic residues in loop D and selectivity of neonicotinoids

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Abbreviations: ACh, acetylcholine; DMSO, dimethyl sulfoxide; nAChR, nicotinic acetylcholine receptor.

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Abstract

The insecticide imidacloprid and structurally-related neonicotinoids act selectively on insect nicotinic acetylcholine receptors (insect nAChRs). To investigate the mechanism of neonicotinoid selectivity, we have examined the effects of mutations to basic amino acid residues in loop D of the nAChR acetylcholine (ACh) binding site on the interactions with imidacloprid. The receptors investigated are the recombinant chicken α4β2 nAChR and *Drosophila melanogaster* Dα2/chicken β2 hybrid nAChR expressed in *Xenopus laevis* oocytes. Although mutations of T77 in loop D of the β2 subunit resulted in a barely detectable effect on the imidacloprid concentration-response curve for the α4β2 nAChR, T77R;E79V double mutations shifted the curve dramatically to higher affinity binding of imidacloprid. Similarly, T77K;E79R and T77N;E79R double mutations in the Dα2β2 nAChR also resulted in a shift to a higher affinity for imidacloprid, which exceeded that observed for a single mutation of T77 to basic residues. By contrast, these double mutations scarcely influenced the ACh concentration-response curve, suggesting selective interactions with imidacloprid of the newly introduced basic residues. Computational, homology models of the agonist binding domain of the wild-type and mutant α4β2 and Dα2β2 nAChRs with imidacloprid bound were generated based on the crystal structures of acetylcholine binding proteins of *Lymnaea stagnalis* and *Aplysia californica*. The models indicate that the nitro group of imidacloprid interacts directly with the introduced basic residues at position 77, whereas those at position 79 either prevent or permit such interactions depending on their electrostatic properties, thereby offering an explanation for the observed functional changes resulting from site-directed mutagenesis.

Introduction

Nicotinic acetylcholine receptors (nAChRs) play a central role in rapid cholinergic synaptic transmission (Sattelle, 1980; Breer and Sattelle, 1990) and are important targets of insecticides (Gepner et al., 1978; Matsuda et al., 2001, 2005). Of the insecticides acting on insect nAChRs, imidacloprid and its analogs (Fig. 1), referred to as neonicotinoids, are used worldwide as agrochemicals (Matsuda et al., 2001, 2005; Tomizawa and Casida, 2005). Also, neonicotinoids are employed in animal health as flea repellants (Mencke and Jeschke, 2002; Rust, 2005). Most neonicotinoids are partial agonists of native (Nagata et al., 1996; Deglise et al., 2002; Ihara et al., 2006) and recombinant (Matsuda et al., 1998 and 2005; Shimomura et al., 2002, 2003 and 2004; Ihara et al., 2003 and 2004) nAChRs, but some antagonize the acetylcholine-induced responses of native insect neurons (Salgado et al., 2004; Ihara et al., 2005) and others show super-agonist actions (Ihara et al., 2004).

Neonicotinoids act selectively on insect nAChRs, accounting at least in part for the selective toxicity to insects over vertebrates (Matsuda et al., 2001, 2005; Tomizawa et al., 2003, 2005). Neonicotinoids possess either a nitro or a cyano group which have been postulated to contribute directly to their selectivity (Matsuda et al., 2001, 2005; Tomizawa et al., 2003, 2005). The molecular targets of neonicotinoids are nAChRs, which belong to the Cys-loop family of ligand-gated ion channels, usually consist of α and non-α subunits (Corringer et al., 2000; Karlin, 2002; Lindstrom, 2003). However, α7, α 8 and α9 subunits each form functional homomers when expressed in *Xenopus* oocytes (Couturier et al., 1990; Elgoyhen et al., 1994; Gerzanich et al., 1994), although α7 (Palma et al., 1999; Azam et al., 2003) and α9 (Elgoyhen et al., 2001) subunits can

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co-assemble with other subunits resulting in characteristics distinct from those of homomers.

The binding site for ACh and other agonists is formed by six loops A-F in the extracellular, N-terminal domain. Loops A-C are confined to α subunits, whereas loops D-F are present either in non-α subunits of α/non-α heteromers, or in the α subunits of homomers (Corringer et al., 2000) and α/α heteromer (e.g. α9/α10) (Elgoyhen et al., 2001).

We have previously shown that when the nitro group of imidacloprid interacts with basic residues, then the nitrogen atoms in the imidazolidine ring of imidacloprid become positive, thereby strengthening cation-π interactions with tryptophan residues in loop B (Matsuda et al., 2001, 2005). This "induced-fit" mechanism can also account for the selective actions of other neonicotinoids such as thiacloprid and acetamiprid, both of which possess a cyano group. Consistent with this view, we have found that Q79R and Q79K mutations in loop D of chicken α7 homomer enhance the peak current amplitude of the currents recorded from the expressed receptor in response to imidacloprid and nitenpyram (Shimomura et al., 2002). Because most insect nAChR non-α subunits possess basic residues in loop D at the position corresponding to Q79 (Residue numbering is from the start methionine) of the α7 subunit (Table 1), it was postulated that such basic residues are likely to contribute to the selective neonicotinoid actions on insect nAChR. Nevertheless, although significant, the shift in the neonicotinoid concentration-response curves resulting from the Q79R and Q79K mutations in the α7 nAChR was small (Shimomura et al., 2002) and insufficient to account for

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neonicotinoid selectivity. Also, it is possible that changes in the responses to neonicotinoids resulting from the addition of basic residues to loop D may be confined to the case for the homomeric α7 nAChRs.

In the present study two-electrode voltage-clamp electrophysiology has been employed to investigate the effects on the responses to imidacloprid of chicken α4β2 and *Drosophila melanogaster* Dα2 (SAD)/chicken β2 hybrid nAChRs of mutating T77 and E79 in the β2 subunit to basic amino acid residues. These residues correspond to Q79 and Y81 of the α7 nAChR (see Table 1). To assist in interpreting the results from mutagenesis experiments, three-dimensional models for the agonist binding site of the nAChRs with imidacloprid docked have been constructed based on the crystal structures of acetylcholine binding proteins (AChBPs) with either nicotine (Celie et al., 2004) or epibatidine (Hansen et al., 2005), which share the pyridine ring with imidacloprid, bound. The models indicated that not only basic residues, but also neighboring structural features, are involved in the selectivity of nAChR-imidacloprid interactions.

Materials and Methods

1. Preparation of DNAs encoding mutant β2 subunits

The chicken nAChR β2 subunit cDNA in the pcDNA3.1 vector (Invitrogen Crop., CA, USA) was used as a template for mutagenesis (Swick et al., 1992; Bertrand et al., 1994). A series of mutations were introduced by PCR as described earlier (Matsuda et al., 2000; Shimomura et al., 2002). Oligonucleotides T77N sense and T77N antisense were prepared to generate the β2 T77N mutation in the β2 subunit. The T7 primer

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(5'-TAATACGACTCACTATAGGGAGACCC-3') and β2 antisense primer (5'-CTTCATTGCAGACCACATGC-3') were designed, respectively, on the basis of the sequence flanking the multiple cloning site of the pcDNA3.1 vector and the β2 cDNA about 1.3 kbp downstream of the start methionine codon. The first round PCRs were carried out using 1 U KOD-Plus polymerase (TOYOBO, Shiga, Japan), 100 ng wild-type pcDNA3.1-β2 as template, 0.3 μM primers (T7 primer and T77K antisense; β2 antisense and T77K sense) and 0.2 mM dNTP mixture in a 50 μl solution for 30 cycles of 94ºC, 15 s; 45ºC, 30 s; 68ºC, 60 s. The second round PCR was conducted using 1 U KOD-Plus, 20 ng each of the first round PCR products and 0.3 μM primers (T7 primer and β2 antisense) and 0.2 mM dNTP mixture in a 50 μl solution for 30 cycles of 94ºC, 15 s; 48ºC, 30 s; 68ºC, 90 s, yielding a single DNA band. After purification using a low melting-point agarose gel (Promega, Madison, WI, USA), the isolated PCR fragment was digested using *Bam*H I (Takara, Shiga, Japan) and subcloned into pcDNA3.1-β2. This plasmid was cut with *Bam*H I and ligated with a 1.3 kbp *Bam*H I fragment of pcDNA3-β2 to complete the full-length mutant β2 subunits. Other DNA constructs encoding the T77N, T77R, E79R, E79V, T77K;E79R, T77N;E79R and T77R;E79V mutants were prepared in a similar manner.

2. Preparation and nuclear injection of Xenopus oocytes

Mature *Xenopus laevis* females were anesthetized by immersion in 1.5 g/l tricaine for 30-45 min, depending on body weight, before removal of a part of the ovary. We made as much effort as possible to minimize animal suffering and reduce the number of animals used. Oocytes at stage V or VI of development were separated from the follicle cell layer after treatment with 2 mg/ml collagenase (Sigma type IA). The

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nucleus of each defolliculated oocyte was injected with 20 nl cDNA in distilled water (0.1 ng/nl) and incubated at 18ºC in standard oocyte saline (SOS) of the following composition (in mM); NaCl 100, KCl 2.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0 and HEPES 5.0, pH 7.6, supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), gentamycin (50 μg/ml) and 2.5 mM sodium pyruvate. Electrophysiology was performed 3-6 days after nuclear injection.

3. Electrophysiology

Xenopus oocytes were secured in a recording chamber which was perfused (7-10 ml/min) continuously with SOS, containing 0.5 μM atropine to eliminate any responses resulting from activation of endogenous muscarinic AChRs, using a gravity-fed delivery system described previously (Matsuda et al., 1998: Shimomura et al., 2004b). Membrane currents were recorded using 2.0 M KCl-filled electrodes (resistances 0.5 - 5.0 MΩ) linked to a GeneClamp 500B (Axon Instruments, Union, CA, USA) amplifier. The oocyte membrane was clamped at -100 mV. Currents were displayed using a pen recorder.

To prepare test solutions, stock solutions of ligands were diluted with SOS containing 0.5 μM atropine, whereas those containing ACh in SOS were prepared immediately prior to experiments. Stock solutions of imidacloprid (300 mM) were prepared in dimethyl sulfoxide (DMSO), and diluted with SOS prior to tests. DMSO at concentrations lower than 1% (v/v) had no effect on the responses (Matsuda et al, 1998). Concentration-response data were obtained by challenging oocytes at intervals of 3-5 min with increasing concentrations of an agonist. The peak amplitude of the current

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recorded in response to each challenge was normalized to the maximum amplitude of the response to ACh. For example, data from the wild-type chicken α4β2 and *Drosophila* Dα2/chicken β2 hybrid nAChRs as well as those from the T77N, T77R, E79R, E79V and T77N;E79R mutants of Dα2β2 nAChR were normalized to the response to 100 μM ACh; data from the T77K, T77N, T77R, T77K;E79R, T77N;E79R and T77R;79V mutants of the α4β2 nAChR, and T77K and T77R;E79V mutants of the Dα2β2 nAChR were normalized to the response to 300 μM ACh; data from the E79R and E79V mutants of the α4β2 nAChR and the T77K;E79R mutant of the Dα2β2 nAChR were normalized to the response to 1 mM ACh.

Using GraphPad 'Prism' (GraphPad Software, San Diego, CA, USA), a non-linear regression analysis was applied to normalize data enabling determination of I_{max}, the maximum normalized response, EC_{50}, the concentration giving half the maximum normalized response and n_H, the Hill coefficient (Matsuda et al., 1998; Shimomura et al., 2002). Experiments were conducted at room temperature (19-25℃). Imidacloprid was synthesized as described (Moriya et al., 1992). ACh chloride and atropine sulfate were purchased from Sigma Aldrich Japan (Tokyo, Japan). .

4. Receptor Modeling

Modeling of the N-terminal domain of chicken α4β2 and *Drosophila* Dα2/chicken β2 hybrid nAChRs were carried out using the molecular modeling software package Sybyl, version 6.91 (Sybyl Molecular Modeling Software, Tripos Associates, Inc., St Louis, MO, USA) and the homology modeling software PDFAMS pro, version 2.0 (Protein Discovery Full Automatic Modeling System; In-Silico Sciences, Inc., Tokyo, Japan),

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originally developed by Ogata and Umeyama (Ogata et al., 2000). Models of the α4β2 nAChR with imidacloprid bound were constructed using PDFAMS ligand & complex mode. Firstly, using PDFAMS, primary sequences of α4 and β2 nAChR subunits obtained from the ligand-gated ion channel database (http://www.ebi.ac.uk/compneur-srv/LGICdb/cyc-loop.php) were automatically aligned with homologous sequences in the FAMS database of PDFAMS, which was constructed using on the PDB database. The alignment included acetylcholine binding proteins for which crystal structures have been obtained with ligands notably of *Lymnaea stagnalis* (Celie et al., 2004) and *Aplysia californica* (Hansen et al., 2005). Because the crystal structures of *L. stagnalis* AChBP complexes had higher resolution (2.2 Å), the snail AChBP bound by nicotine (1UW6) (Celie et al., 2004) was basically selected as the reference protein. However, imidacloprid is similar in structure to epibatidine and the only available structure of AChBP bound by epibatidine is from *A. californica*. Therefore, the crystal structure of *A. californica* AChBP-epibatidine complex (2BYQ) was superimposed on the structure of *L. stagnalis* AChBP bound by nicotine (1UW6). Then imidacloprid was superimposed on the structure of epibatidine to determine the coordinates of the insecticide.

In the second step, the three dimensional structures of α4β2 nAChR-imidacloprid complex were constructed based on the sequence and coordinates of AChBP and the coordinates of imidacloprid by the simulated annealing method (Kirkpatrick, 1983). The coordinates of imidacloprid were fixed during the simulated annealing. Since water molecules may play an important role for the ligand-receptor interaction, water molecules were set in the α4β2 ligand binding site at the same coordinates as those of

1UW6 (after superposition of the complex model on 1UW6). The receptor model constructed in this way was energy-minimized for 1,000 iterations of conjugated gradients using the force field and partial charges of the molecular mechanics MMFF94 (Halgren 1999a, b). The structure was then subjected to molecular dynamics simulation, where it was 10 cycles of heated to 700° K and annealed slowly to 200° K. The last structure from the molecular dynamics trajectory was energy-minimized again for 1,000 iterations of conjugated gradients using the force field and partial charges of MMFF94. The ligand-receptor complex with water molecules was minimized using a procedure similar to that outlined above. The model of the wild-type *Drosophila* Dα2/chicken β2 nAChR bound by imidacloprid was constructed in a similar manner to that of α4β2 nAChR-imidacloprid complex. In mutant receptor models, T77 and E79 of the β2 subunit were replaced by the mutated residues in the alignment. Subsequent procedures were the same as employed for the wild-type proteins.

Results

In voltage-clamp electrophysiological studies, ACh and imidacloprid evoked inward currents in a dose-dependent manner in *Xenopus* oocytes expressing the wild-type and mutant α4β2 and Dα2β2 nAChRs. The concentration-response curves for ACh and imidacloprid for the wild-type α4β2 and Dα2β2 nAChRs, newly measured as controls in this study, resemble closely those previously reported (Shimomura et al., 2003 and 2005). The I_{max} (= normalized maximum response) and pEC_{50} (=log ($1/EC_{50}$ (M)) values of ACh for the non-mutated α4β2 nAChR were 1.18 ± 0.04 and 5.14 ± 0.07 (n=7), respectively, whereas the I_{max} and pEC_{50} values for the wild-type Dα2β2 nAChR were 1.02 ± 0.02 and 4.76 ± 0.03 (n=7), respectively (Figure 2, Table 2). The

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responses to imidacloprid of the wild-type α4β2 nAChR were too small to obtain the I_{max} and pEC_{50} values, whereas imidacloprid activated the wild-type Dα2β2 nAChR with I_{max} and pEC_{50} values of 0.55 ± 0.03 and 5.45 ± 0.08 (n=4), respectively (Figure 2, Table 2).

The pEC_{50} value of ACh for the α4β2 nAChR was minimally shifted by T77K (5.08 ± 0.13 (n=5), T77N (5.47 ± 0.12 (n=5)), T77R (4.83 ± 0.12 (n=6)), E79R (4.86 ± 0.16 (n=4)) and E79V (4.76 ± 0.05 (n=4)) mutations (Figures 2A and 2E, Table 2). Similarly, imidacloprid failed to activate the α4β2 nAChR irrespective of the presence or absence of such single amino acid replacements in loop D (Figures 2B and 2F, Table 2).

When T77 in loop D of the β2 subunit was replaced by lysine in the Dα2β2 nAChR, the ACh concentration-response curve was shifted to the right (pEC_{50} = 4.56 ± 0.03 (n=6)) (Figure 2C, Table 2). However, this was unique, and all other changes in the concentration-response curve of ACh for the Dα2β2 nAChR following a single amino acid mutation at position of 77 or 79 in loop D of the β2 subunit were minimal: Dα2β2 (T77N) mutant, 4.88 ± 0.04 (n=5); Dα2β2 (T77R) mutant, 4.83 ± 0.04 (n=5); Dα2β2 (E79R) mutant, 4.70 ± 0.05 (n=5); Dα2β2 (E79V) mutant, 4.75 ± 0.04 (n=6) (Figures 2C and 2G, Table 2). On the other hand, T77N or T77R mutation in loop D of the β2 subunit resulted in significant shifts of the imidacloprid concentration-response curve to the left (pEC_{50} values of imidacloprid for the T77N and T77R mutants of Dα2β2 nAChR were 6.10 ± 0.12 (n=4) and 6.11 ± 0.12 (n=7), respectively) (Figure 2D, Table 2). In all other cases, the concentration-response curves for the Dα2β2 nAChR of

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imidacloprid resulting from single amino acid replacements were similar to those observed for Dα2β2 wild-type nAChR (Figures 2D and 2H, Table 2).

To help interpret these results, three-dimensional models of the ligand binding site of the α4β2 and Dα2β2 nAChRs bound by imidacloprid were constructed (Figure 3). The wild-type models (Figures 3A and 3B) show T77 in the proximity to the nitro group of imidacloprid. Comparing α4β2 and Dα2β2 nAChR models indicates stronger contact with the nitro group of the insecticide in the case of the hybrid nAChR. In both cases, E79 appears to indirectly influence the interactions of T77. Thus, mutation of T77 and E79 is predicted to influence imidacloprid interactions with both nAChRs under investigation. Figures 3C and 3D show that T77R;E79V double mutations in the α4β2 and Dα2β2 nAChRs can assist in the electrostatic interactions of the basic residue with the nitro group of imidacloprid, thereby enhancing markedly agonist affinity. Therefore, we have also investigated the effects of combined mutations of T77 and E79 in loop D to residues observed in the insect non-α subunits on the responses to imidacloprid of the α4β2 and Dα2β2 nAChRs.

Compared with the effects of the single amino acid mutations, the effects of double amino acid mutations at positions 77 and 79 were striking: The T77R;E79V double mutant of the α4β2 nAChR showed greatly enhanced responses to imidacloprid (Figures 4A and 4B). The pEC_{50} and Imax values of imidacloprid for the α4β2 (T77K;E79R) mutant were 4.50 ± 0.18 and 0.53 ± 0.06 (n=6), respectively (Table 2).

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The T77K;E79R, T77N;E79R and T77R;E79V mutations of the Dα2β2 nAChR shifted the imidacloprid concentration-response curve dramatically to the left (Figures 4C, 4D and 5D). The pEC_{50} values of imidacloprid for the T77K;E79R, T77N;E79R and T77R;E79V mutants were 5.87 ± 0.07, 6.58 ± 0.06 and 6.19 ± 0.12, respectively (Table 2). The I_{max} values obtained from the dose-response curve of imidacloprid for the T77K;E79R, T77N;E79R and T77R;E79V mutants were 0.63± 0.03 (n=5), 0.65 ± 0.02 (n=6) and 0.44 ± 0.03 (n=8), respectively (Table 2). By contrast, for both receptor types, the dose-response curves for ACh were much less affected by the double mutations in loop D of the β2 subunit (Figures 5A and 5C, Table 2).

Discussion

In this study, we have shown that amino acid substitutions in loop D designed to mimic insect non-α subunits greatly increases the affinity of imidacloprid in terms of the pEC_{50} value for the α4β2 and Dα2β2 nAChRs, whereas the impact on ACh concentration-response curve is much smaller. This points to an important role of loop D in the selectivity of imidacloprid for the recombinant nAChRs investigated.

To enhance significantly the imidacloprid-sensitivity of the α4β2 nAChR in terms of the shift of pEC_{50} value, it was necessary to replace not only T77 by basic residue, but also E79 by neutral residue valine. An interpretation of this result is that the electrostatic force of R77 enhancing the α4β2 nAChR-imidacloprid interactions is suppressed by the negative electrostatic force of E79 only when T77R mutation was added to loop D. However, this “lock” is removed by the E79V mutation, resulting in an enhancement of the current amplitudes in response to imidacloprid. The shift of the

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imidacloprid concentration-response curve for the α4β2 and Dα2β2 nAChRs by the T77K;E79R double mutation was smaller than that induced by the T77R;E79V double mutation. In view of the result that the ACh concentration-response curves were shifted by the T77K;E79R mutation to higher concentrations irrespective of the kinds of nAChRs tested (Table 2), this mutation might alter the conformation of the agonist binding site to reduce the affinity of all agonists, thereby counteracting the enhancement of the affinity for imidacloprid by the basic residues.

We have generated three-dimensional models of wild-type and mutant α4β2 and Dα2β2 nAChR bound by imidacloprid to help understand the mechanism underlying the results following the site-directed mutagenesis (Figure 3). The locations of key amino acid residues (Y121 (loop A), W177 (loop B), Y218 (loop C), C220 (loop C), C221 (loop C), and Y225 (loop C) of the α4 subunit; and W75 (loop D) and F137 (loop E) of the β2 subunit; residue numbering is from the start methionine) in the models resembled those located in the model reported earlier shown by Novère et al. (2002). The nitro group appears to be located apart from T77 in the wild-type α4β2 nAChR model (Figure 3A), whereas the nitro group oxygens contact with R77 in the T77R;E79V mutant model (Figure 3C), consistent with the shift to lower concentrations of the imidacloprid concentration-response curve following the T77R;E79V double mutation (Figures 4 and 5). Similarly, the T77R;E79V double mutation, which resulted in a significant increase of the pEC_{50} value of imidacloprid for the Dα2β2 hybrid nAChR (Figures 4, 5), placed the nitro group in contact with R77 in the model (Figure 3D). Thus, the enhancement of the recombinant AChRs following the double site-directed mutagenesis is likely due to direct electrostatic interactions of the nitro group of imidacloprid with the basic

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residues in loop D.

The Dα2β2 hybrid nAChR was more sensitive to imidacloprid than the α4β2 nAChR in terms of the shift of the imidacloprid concentration-response curve, suggesting that the Dα2 subunit possesses structural features favoring interactions with imidacloprid. This agrees well with earlier findings that the region upstream of loop B and an amino acid in loop C also play a role in determining selectivity (Shimomura et al., 2004a, 2005). However, the contribution of the non-α subunit β2 to the interactions with imidacloprid should not be undervalued, as demonstrated earlier by Lansdell and Millar (2000). The molecular modeling (Figure 3B) showed closer proximity of the nitro group to T77 in the Dα2β2 nAChR compared with its position in the α4β2 nAChR (Figure 3A), thereby facilitating hydrogen bond formation between the ligand and the hybrid nAChR. Consistent with the models, not only T77N;E79R and T77R;E79V double mutations, but also T77N and T77R point mutations significantly increased the pEC_{50} value of imidacloprid in the Dα2β2 nAChR, which was not observed for the α4β2 nAChR. All these results seem to suggest that the amino acids newly introduced to position 77 in loop D are able to interact more strongly with the nitro group of imidacloprid in the Dα2β2 nAChR than in the α4β2 nAChR, thereby resulting in the significant shifts of the imidacloprid concentration-response curve to lower concentrations (Table 2). However, the high imidacloprid sensitivity of the Dα2β2 nAChR may stem partly from the interactions of one of the two nitro group oxygens with other regions than loop D. Thus further studies are necessary to fully understand the mechanism for the selectivity of neonicotinoids.

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The EC_{50} value of imidacloprid in the T77N;E79R mutant of the Dα2β2 nAChR is lower than 1 μM, which is close to the value determined using the cockroach (*Periplaneta americana*) native neurons (Ihara et al., 2006). Therefore, the overall difference in imidaloprid sensitivity between insect and mammalian nAChRs *in vivo* can be accounted for by the interactions with loop D in the non-α subunit combined with those with loop C and the region upstream of loop B in the α subunit of insect nAChRs (Shimomura et al., 2004a, 2005).

In conclusion, we have shown that the basic residues observed only in insect nAChR loop D play a key role in the selective interactions of heteromeric nAChRs with neonicotinoids. It is conceivable that the double mutations in loop D enhance markedly the imidacloprid sensitivity of not only α4β2 and Dα2β2 nAChRs tested in this study, but also other nAChRs. Molecular modeling has been employed, based on the structure of AChBP with nicotine bound and this accounts well for the experimental data obtained by site-directed mutagenesis. It has been found recently that a mutation in loop B leads to a neonicotinoid resistance in brown leaf hoppers (Liu et al., 2005). In future the model developed here could be exploited further to examine the molecular basis of neonicotinoid resistance and to study the docking of newly developed insecticide candidates with nAChRs. In the context of receptor-based neonicotinoid resistance, our findings indicate that substitutions to non-basic residues in two key sites in loop D may also lead to a neonicotinoid resistant phenotype because the effects of such structural changes on the ACh concentration-response curve are minimal. The present study contributes to our understanding of the molecular mechanism underlying selectivity of neonicotinoids and suggests a possible target region on which to focus in

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the design of new insecticides.

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Footnotes

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Figure Legends

Figure 1 Chemical structures and electrostatic potentials of acetylcholine (A) and imidacloprid (B). The electrostatic potentials were calculated by the MNDO, semi-empirical, molecular orbital method. Positive and negative potentials are shown in red and blue, respectively. (C) Neonicotinoid insecticides which have been developed to date following the discovery of imidacloprid.

Figure 2 Concentration-response curves of acetylcholine (ACh) (A, C, E and G) and imidacloprid (IMI) (B, D, F and H) obtained for wild-type, T77K, T77N and T77R mutants of the α4β2 (A and B) and Dα2β2 (C and D), and wild-type, E79R and E79V mutants of the α4β2 (E and F) and Dα2β2 (G and H) nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. Each plot represents mean ± standard error of the mean of 4-8 experiments.

Figure 3 Homology models of the agonist binding domain of the wild-type α4β2 (A) and Dα2β2 (B) nAChRs and their T77R;E79V mutants (C, α4β2 nAChR; D, Dα2β2 nAChRs) bound by imidacloprid constructed using the crystal structure (1UW6) of the acetylcholine binding protein(AChBP) from snail *Lymnaea stagnalis.* The figures were made using Sybyl version 6.91 and PDFAMS (See Materials and Methods for details). In the wild type nAChR models, only imidacloprid, T77 and E79 are shown by the space-filling model, whereas in the mutant nAChR models, only imidacloprid, R77 and V79 are shown (main chain of the α4 and Dα2 subunits, orange; main chain of the β2 subunit, green; carbon, white; hydrogen, cyan; nitrogen, blue; oxygen, red; chlorine, green blue). Enlarged views are shown under respective whole views.

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Figure 4 Inward currents recorded using two electrode voltage-clamp electrophysiology in response to bath-applied acetylcholine (ACh) and imidacloprid (IMI) of the wild-type α4β2 nicotinic acetylcholine receptor (nAChR) (A) and its T77R;E79V mutant (B), and the wild-type Dα2β2 nAChR (C) and its T77N;E79R mutant (D) expressed in *Xenopus laevis* oocytes.

Figure 5 Concentration-response curves of acetylcholine (ACh) (A and C)) and imidacloprid (IMI) (B and D) obtained for wild-type, T77K;E79R, T77N;E79R and T77R;E79V mutants of the α4β2 (A and B) and Dα2β2 (C and D) nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. Each plot represents mean ± standard error of the mean of 4-8 experiments

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Table 1. Alignment of amino acid sequence in loop D region of nicotinic acetylcholine receptors of vertebrates and insects.

	Subunits	Amino acid number of chicken β2 subunit*									
		73	74	75	76	77	78	79	80	81	82
Vertebrates	Chicken α7	N	I	W	L	Q	M	Y	W	T	D
	Chicken β2	N	V	W	L	T	Q	E	W	E	D
	Chicken β4	N	V	W	L	N	Q	E	W	I	D
	Human β2	N	V	W	L	T	Q	E	W	E	D
	Human β4	N	V	W	L	K	Q	E	W	T	D
	Human δ	N	V	W	I	E	H	G	W	T	D
	Human ε	S	V	W	I	G	I	D	W	Q	D
	Rat β1	K	V	Y	L	D	L	E	W	T	D
	Rat β2	N	V	W	L	T	Q	E	W	E	D
	Rat β4	S	I	W	L	K	Q	E	W	T	D
Insects	Fruit fly SBD	N	L	W	V	K	Q	R	W	F	D
	Fruit fly β3	H	C	W	L	N	L	R	W	R	D
	Fruit fly ARD	N	V	W	L	R	L	V	W	Y	D
	Locust migratoria β	N	V	W	L	R	L	V	W	N	D
	Myzus persicae β1	N	V	W	L	R	L	V	W	R	D
	Heliothis virescens β1	N	V	W	L	R	L	V	W	M	D

*Residue numbering is from the start methionine.

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Table 2. I_{max}, EC_{50} and n_H values of acetylcholine and imidacloprid for wild-type and mutant α4β2 and Dα2β2 nicotinic acetylcholine receptors

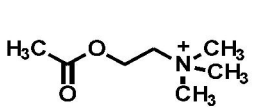
	Acetylcholine			Imidacloprid		
	I_{max}	pEC_{50}	n_H	I_{max}	pEC_{50}	n_H
α4β2 Wild-Type	1.18 ± 0.04	5.14 ± 0.07	0.6 ± 0.1	nd[a]	nd	nd
	1.17 ± 0.05[b]	5.12 ± 0.09[b]	0.6 ± 0.1[b]	nd[b]	nd[b]	nd[b]
α4β2 T77K	1.12 ± 0.07	5.08 ± 0.13	0.6 ± 0.1	nd	nd	nd
α4β2 T77N	1.06 ± 0.06	5.47 ± 0.12	0.6 ± 0.1	nd	nd	nd
α4β2 T77R	0.99 ± 0.06*	4.83 ± 0.13	0.7 ± 0.1	nd	nd	nd
α4β2 E79R	1.01 ± 0.07	4.86 ± 0.16	0.6 ± 0.1	nd	nd	nd
α4β2 E79V	1.03 ± 0.05	4.76 ± 0.10	0.7 ± 0.1	nd	nd	nd
α4β2 T77K;E79R	1.09 ± 0.03	4.52 ± 0.06**	0.9 ± 0.1	0.07 ± 0.02	3.67 ± 0.25	1.4 ± 0.8
α4β2 T77N;E79R	1.00 ± 0.04	5.02 ± 0.08	0.9 ± 0.1	nd	nd	nd
α4β2 T77R;E79V	1.02 ± 0.04	5.39 ± 0.08	0.8 ± 0.1	0.53 ± 0.06	4.50 ± 0.18	0.9 ± 0.3
Dα2β2 Wild-Type	1.02 ± 0.02	4.76 ± 0.03	1.5 ± 0.1	0.55 ± 0.03	5.45 ± 0.08	1.4 ± 0.3
	1.00 ± 0.03[b]	4.81 ± 0.05[b]	1.5 ± 0.2[b]	0.62 ± 0.02[b]	5.83 ± 0.05[b]	1.7 ± 0.3[b]
Dα2β2 T77K	0.97 ± 0.02	4.56 ± 0.03**	1.5 ± 0.1	0.53 ± 0.03	5.88 ± 0.08	1.4 ± 0.3
Dα2β2 T77N	1.07 ± 0.03	4.88 ± 0.04	1.1 ± 0.1	0.37 ± 0.03*	6.10 ± 0.12*	1.2 ± 0.3
Dα2β2 T77R	1.08 ± 0.03	4.83 ± 0.04	1.1 ± 0.1	0.43 ± 0.03	6.11 ± 0.12**	1.1 ± 0.3
Dα2β2 E79R	1.06 ± 0.04	4.70 ± 0.05	1.5 ± 0.2	0.38 ± 0.04*	5.89 ± 0.16	1.1 ± 0.4
Dα2β2 E79V	1.08 ± 0.03	4.75 ± 0.04	1.4 ± 0.1	0.54 ± 0.05	5.52 ± 0.15	0.8 ± 0.2
Dα2β2 T77K;E79R	0.96 ± 0.02	4.19 ± 0.03**	1.8 ± 0.2	0.63 ± 0.03	5.87 ± 0.07	1.3 ± 0.3
Dα2β2 T77N;E79R	1.09 ± 0.03	4.67 ± 0.04	1.4 ± 0.1	0.65 ± 0.02	6.58 ± 0.06**	1.5 ± 0.2
Dα2β2 T77R;E79V	1.00 ± 0.03	4.49 ± 0.05**	1.3 ± 0.2	0.44 ± 0.03	6.19 ± 0.12**	1.0 ± 0.3

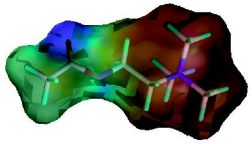
Values shown are the result of a fit of the concentration-response data (mean ± standard error of the mean, 4-8) illustrated in Figs. 2 or 4. Statistical test (one-way ANOVA, Dunnett's multiple comparison test) is for significant differences from the wild-type data (*<0.05; **<0.01).

[a]Abbreviations: nd, not determined because the concentration-response curve did not plateau even at 1 mM.

[b]Data were cited from Shimomura et al., 2005.

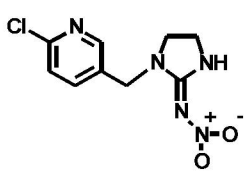
A

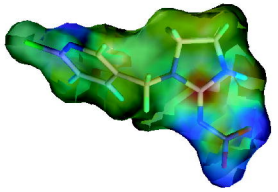




Acetylcholine

B





Imidacloprid

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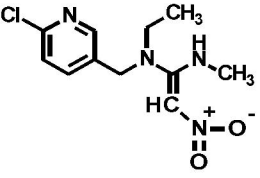

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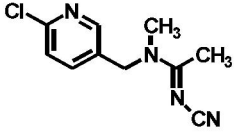
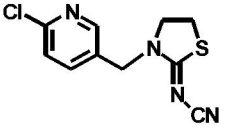
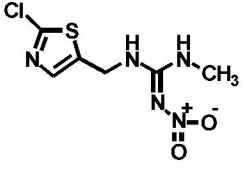


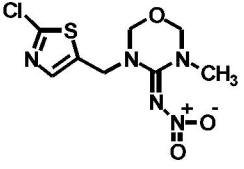
Acetamiprid



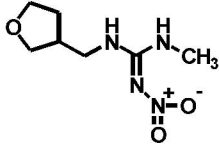
Thiacloprid



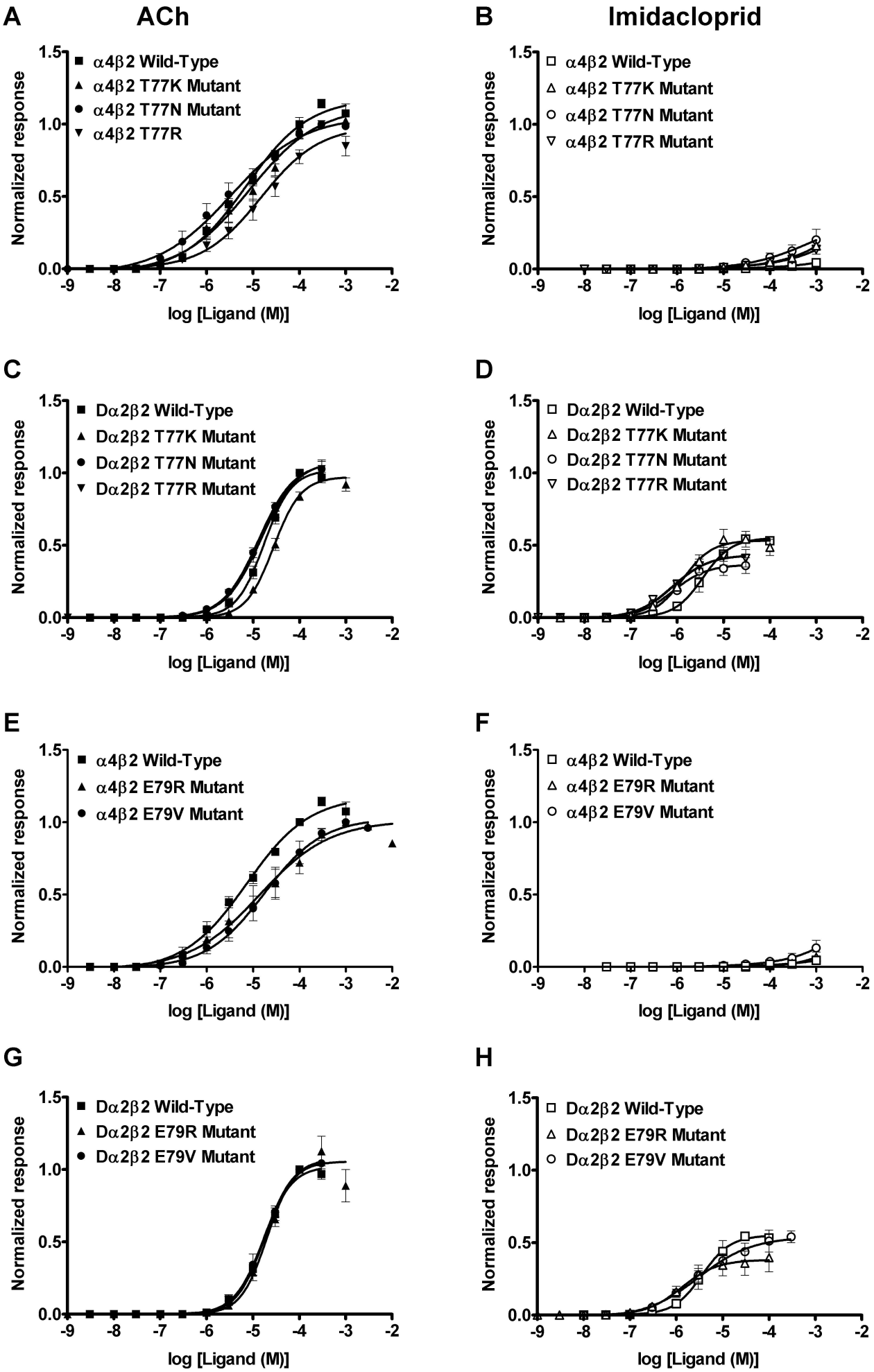
Clothianidin

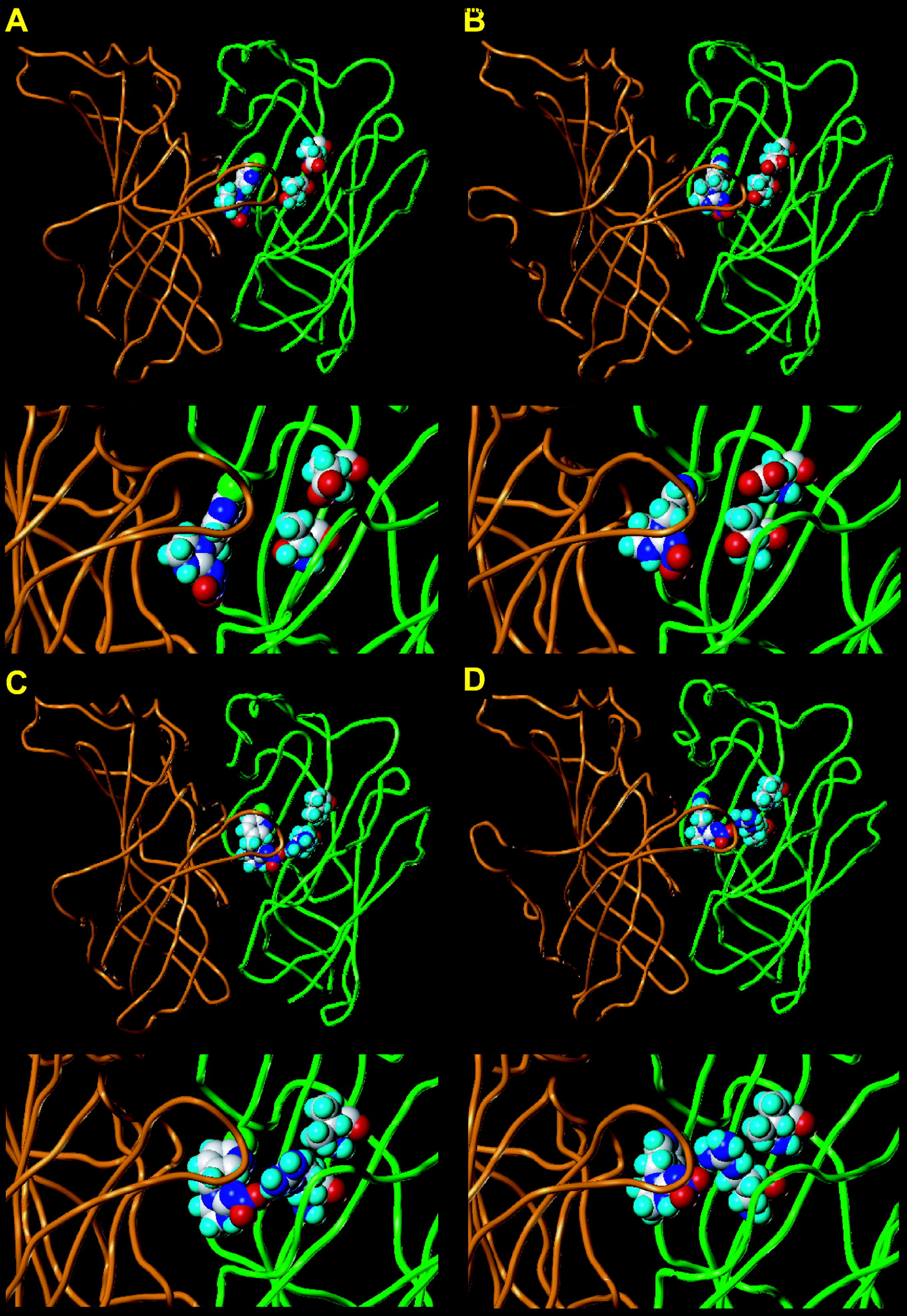


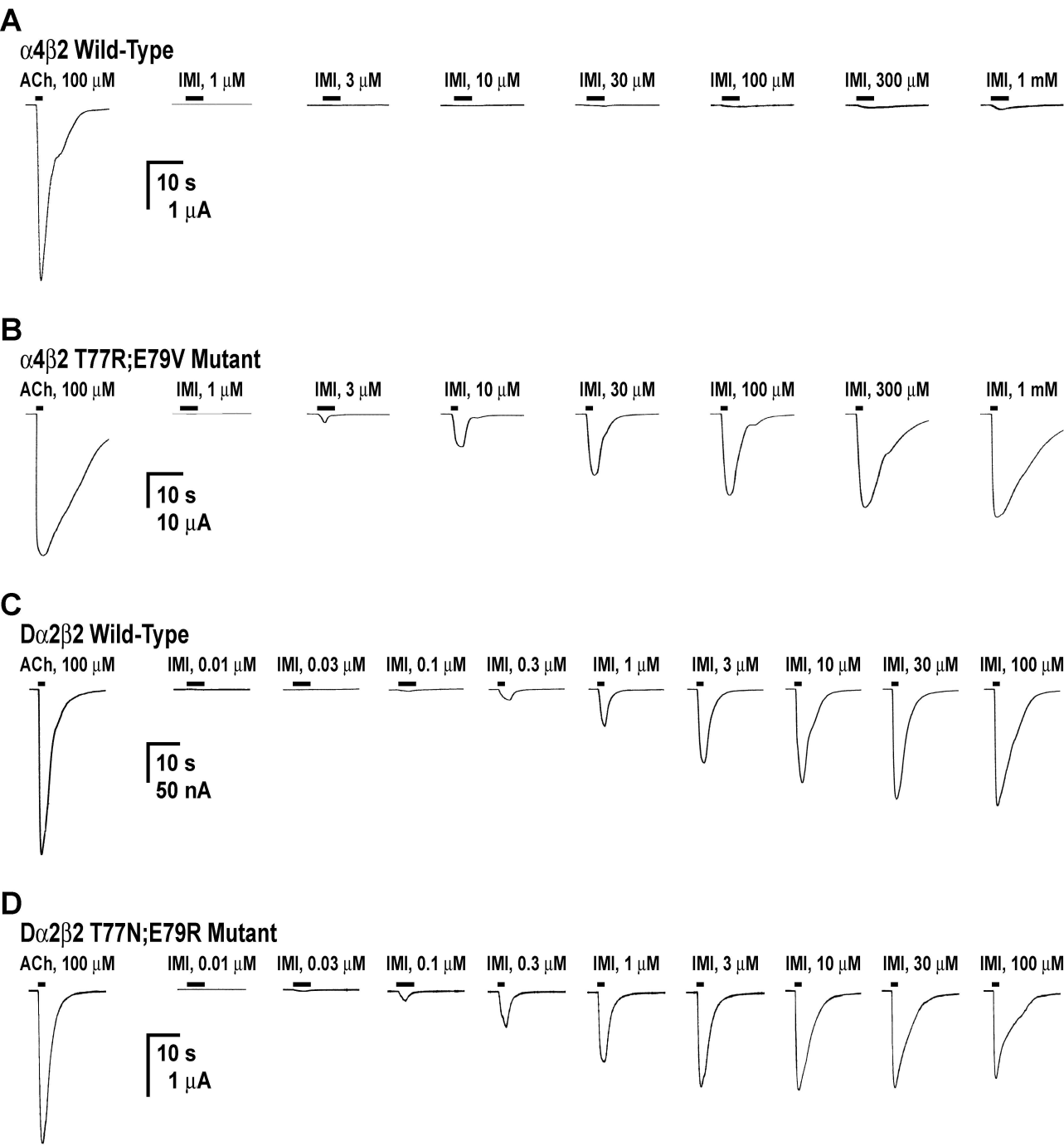
Thiamethoxam

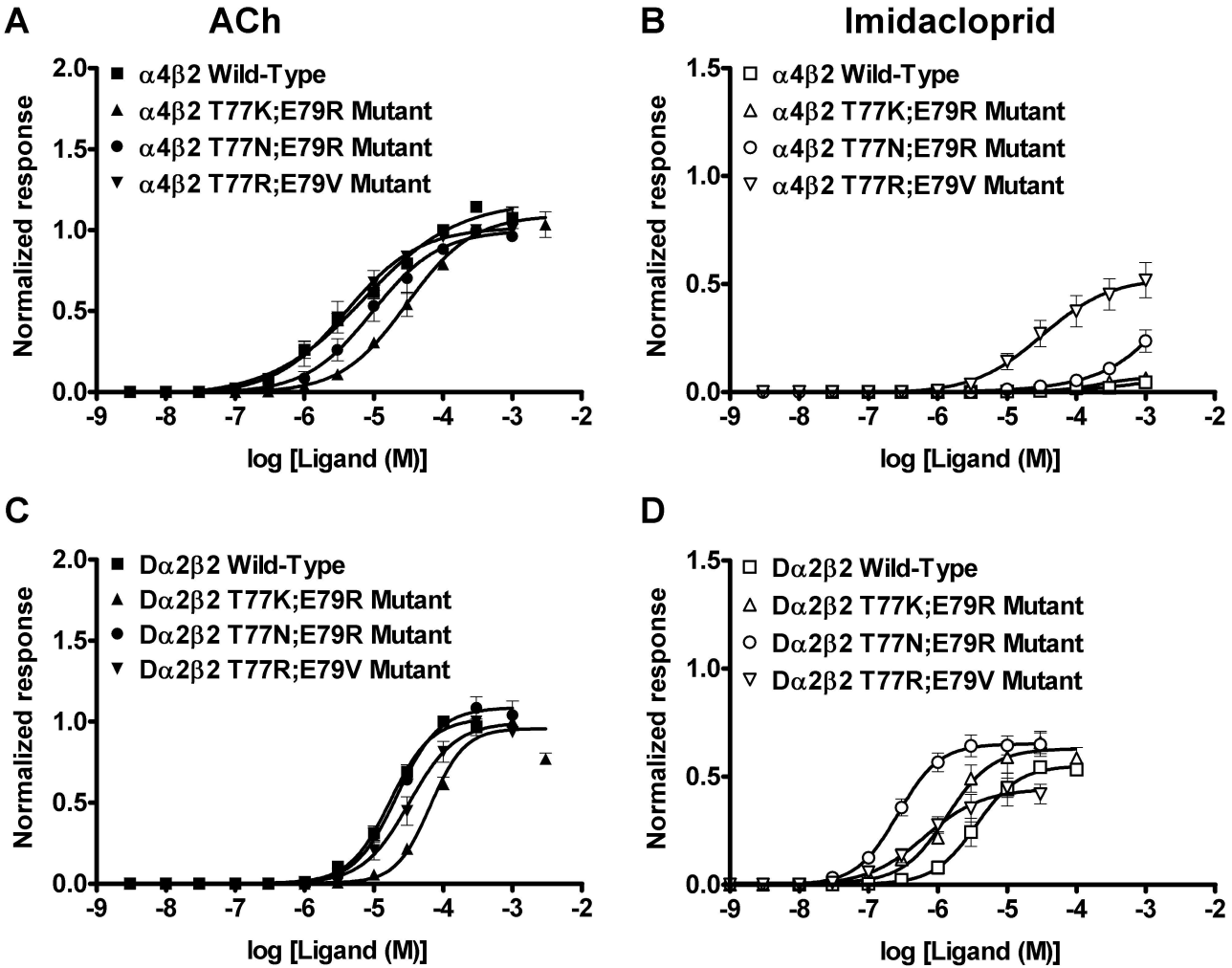


Dinotefuran


A
ACh
B
Imidacloprid
C
D
E
F
G
H
α4β2 Wild-Type
α4β2 T77K Mutant
α4β2 T77N Mutant
α4β2 T77R
α4β2 T77R Mutant
Dα2β2 Wild-Type
Dα2β2 T77K Mutant
Dα2β2 T77N Mutant
Dα2β2 T77R Mutant
α4β2 E79R Mutant
α4β2 E79V Mutant
Dα2β2 E79R Mutant
Dα2β2 E79V Mutant
Normalized response
log [Ligand (M)]
1.5
1.0
0.5
0.0
-9
-8
-7
-6
-5
-4
-3
-2


A
B
C
D


A
α4β2 Wild-Type
ACh, 100 μM
IMI, 1 μM
IMI, 3 μM
IMI, 10 μM
IMI, 30 μM
IMI, 100 μM
IMI, 300 μM
IMI, 1 mM
10 s
1 μA
B
α4β2 T77R;E79V Mutant
ACh, 100 μM
IMI, 1 μM
IMI, 3 μM
IMI, 10 μM
IMI, 30 μM
IMI, 100 μM
IMI, 300 μM
IMI, 1 mM
10 s
10 μA
C
Dα2β2 Wild-Type
ACh, 100 μM
IMI, 0.01 μM
IMI, 0.03 μM
IMI, 0.1 μM
IMI, 0.3 μM
IMI, 1 μM
IMI, 3 μM
IMI, 10 μM
IMI, 30 μM
IMI, 100 μM
10 s
50 nA
D
Dα2β2 T77N;E79R Mutant
ACh, 100 μM
IMI, 0.01 μM
IMI, 0.03 μM
IMI, 0.1 μM
IMI, 0.3 μM
IMI, 1 μM
IMI, 3 μM
IMI, 10 μM
IMI, 30 μM
IMI, 100 μM
10 s
1 μA


A
ACh
Normalized response
log [Ligand (M)]
α4β2 Wild-Type
α4β2 T77K;E79R Mutant
α4β2 T77N;E79R Mutant
α4β2 T77R;E79V Mutant
B
Imidacloprid
Normalized response
log [Ligand (M)]
α4β2 Wild-Type
α4β2 T77K;E79R Mutant
α4β2 T77N;E79R Mutant
α4β2 T77R;E79V Mutant
C
Normalized response
log [Ligand (M)]
Dα2β2 Wild-Type
Dα2β2 T77K;E79R Mutant
Dα2β2 T77N;E79R Mutant
Dα2β2 T77R;E79V Mutant
D
Normalized response
log [Ligand (M)]
Dα2β2 Wild-Type
Dα2β2 T77K;E79R Mutant
Dα2β2 T77N;E79R Mutant
Dα2β2 T77R;E79V Mutant